## REMARKS

Upon entry of the foregoing amendments, claims 1-27, 29,37, 47 and 48 will be pending. Claims 1, 34 and 36 are the only independent claims.

For the following reasons, this Amendment and the accompanying Langer Declaration (with curriculum vitae as Appendix A and references) and the Request for Continued Examination are believed to overcome the rejections of record in the Office Action of March 11, 2004, containing a final rejection of this application, which was the subject of a Notice of Appeal that is now moot in consideration of these documents.

Initially, Applicants are pleased to note that all prior art rejections have been overcome or withdrawn based on the prior prosecution of this application, and that priority of this application under 35 U.S.C. § 119 has been accorded to Applicants' European application No. EP 97 10 0012.0, filed January 2, 1997 (the "prior European application"). Although in section 5 at page 2 of the Detailed Action the Examiner advised Applicants to amend the first line of the specification to claim the benefit of such foreign application, Applicants' respectfully submit that the claim of the benefit of foreign priority and a specific reference to the foreign application need not be made directly in the specification, as the Cross-Reference to Related Applications are appropriate only for claiming the benefit of earlier priority dates of related applications that are continuations, divisions, continuations-in-part, applications under 35 U.S.C. § 371, and provisional U.S. applications. The benefit of the prior European application has been claimed through the appropriate documentation in the file history of the present application.

Claim 49, directed to a medicament comprising the nucleic acid molecule of claim 1, has been cancelled without prejudice. Thus, the basis for the rejections in the first two paragraphs at page 3 of the Detailed Action are now moot.

Many of the claims have been amended to place them in better form for allowance and to clarify the invention, all without adding new matter. Claim1 has been amended to delete recitations of fragments or derivatives of the effector and processing modules of the mistletoe lectin chain A and the mistletoe lectin propeptide, respectively, and to include conventional hybridization conditions for appropriate nucleic acid molecules which hybridize with nucleic acid molecules recited in portions (a) (i) and (ii) and (b) (i) and (ii) of the claim. The deletion of fragments and derivatives is believed to overcome the basis of rejections of this claim and those claims dependent on claim 1. The

inclusion of the conventional hybridization conditions is supported by the application as filed at page 16, lines 17-25, and is specifically intended to include equivalent hybridization conditions as would be known to one skilled in this art, with reference, for example, to the Sambrook, *et al.* and Hames and Higgins texts cited in this section of the specification. Likewise, conventional hybridization conditions have been recited in claims 5, 34 and 36.

Attached as Exhibit 1 is a copy of the title page of Sambrook, *et al.*, and pages 2.114-2.117, 9.47-9.51 and 11.44-11.49. These pages relate to conventional hybridization conditions and make it clear that one skilled in the art would understand that hybridization temperature is a function of the length of the oligonucleotide probe. The hybridization temperature is about 20 to about 25° C below the melting temperature, which for oligonucleotides longer than 100 bases can be calculated according to the formula at page 9.51. The suggested hybridization temperature is 68° C (page 2.114, item 3). The melting temperature for shorter oligonucleotides having a length between 14 and 17 bases can be calculated according to the formula at page 11.46, resulting in a suggested hybridization temperature of about 48 to about 66° C, depending on the length (page 11.48). These calculations were used to determine the hybridization temperatures recited in the claims. Thus, the hybridization temperature in claims 1(a)(iii) and 34(a)(iii) is about 65° C (in line with the temperature at page 2.114, item 3), and the hybridization temperature in claims 1(b)(iii), 5(iii) and 36(b)(iii) is about 50° C. In view of this explanation, Applicants respectfully submit that the amendments are fully supported and no new matter has been added.

Claim 8 has been amended to clarify what S3 and S4 are affirmatively, rather than merely negatively. This amendment is supported in the specification at page 23, lines 8-9.

Claims 10 and 11 have been amended to make it clear that the cells involved participate in acquired immunity, consistent with the understanding of one skilled in the art that the specific immune system is also understood as the acquired immune system, as will be explained below with respect to the Langer Declaration.

Claim 13 has been amended to make it clear that the cells involved participate in innate immunity, consistent with the understanding of one skilled in the art that the unspecific immune system is also understood as the innate immune system, as also will be explained below with respect to the Langer Declaration.

Claim 15 has been amended to correct an obvious typographical error with respect to punctuation in the SEQ ID NO.

Claim 17 has been amended to delete a recitation of an exchange at position 79, since that position is recited in claim 47.

Claims 19 and 20 have been amended to correct an obvious grammatical error in each by removing an unnecessary "is" from the claims.

Claim 22 has been amended to include a period at the end of the claim.

Claim 29 has been amended to consistently refer to the method as a "method," rather than as both a "process" and a "method," even though such terms are synonyms.

Independent claim 34, similarly to claim 1, has been amended to delete references to fragments and derivatives of the effector module and to recite conventional hybridization conditions as appropriate.

Claim 35, depending from claim 34, has been amended to delete a reference to a derivative of the mistletoe lectin propeptide.

Independent claim 36, similarly to claims 1 and 34, has been amended to delete references to fragments and derivatives of the processing module and to recite conventional hybridization conditions as appropriate.

Claim 37, depending from claim 36, has been amended to delete a reference to a derivative of mistletoe lectin A.

Claim 47 has been amended to include a recitation to position 79, moved from claim 17, since claim 48 refers to a preferred substitution at this position.

Claim 48 has been amended to correct the typographical error relating to the claim from which it depends to clarify that claim 48 depends from claim 47, rather than from claim 37.

Since all of the claim amendments are fully supported by the application as filed, no new matter has been added and their entry is respectfully solicited.

In section 8 at pages 2-4 of the Detailed Action, claims 9, 10, 12-14, 16, 24 and 25 were rejected under 35 U.S.C. § 112, first paragraph, as not being supported by an enabling disclosure. In considering Applicants' prior arguments, the Examiner mentioned that they appeared to suggest a method of trial and error without a particular expectation of success with a any particular construct, thus comprising undue experimentation. In section 9 at pages 4-5 of the Detailed Action, all of the pending claims were rejected under 35 U.S.C. § 112, first paragraph, as not being supported by a written description in the specification. The Examiner apparently is of the opinion that Applicants have not provided evidence that they were in possession of a representative number of what the

Examiner considered an "essentially limitless number of polynucleotides encoding fusion proteins ...." In view of the amendments and the following reasons, including the Langer Declaration and its references, Applicants respectfully disagree with and traverse, and also request reconsideration and withdrawal of the rejections under 35 U.S.C. § 112, first paragraph.

Applicants have amended several claims as discussed above to delete recitations of fragments and derivatives of modules of the nucleic acid molecule claimed in this application, and have included conventional hybridization conditions for certain of the modules, as well. These amendments, together with the explanation in paragraphs 13-21 of the Langer Declaration and the references cited therein. Dr. Langer is well-qualified in the field of molecular biology, and is familiar with this application, as well as the amended claims, as noted in paragraphs 2-12 of the Langer Declaration. While he is not an inventor of the subject matter claimed in this application, he has been employed by both the prior assignee and the present assignee of this invention (see Langer Declaration paragraphs 4, 5 and 8), and is representative of a person having a high level of skill in the art of molecular biology, cytology and immunology, to whom the present application is directed (see Langer Declaration paragraphs 8-12).

As Dr. Langer explains in paragraphs 13-20 of the Langer Declaration, one skilled in the art, in view of the disclosures in the present application, would certainly understand and appreciate how to make and use the claimed invention and would also readily appreciate that Applicants were in possession of the claimed invention in sufficient detail and in a sufficient representative number of examples. The references cited by Dr. Langer in these paragraphs and provided with the Langer Declaration, together with Dr. Langer's explanations and citations to the application, provide clear evidence for the conclusion in paragraph 21 of the Langer Declaration that one skilled in the art would be enabled by the application and would realize that Applicants possessed their invention at the time it was filed.

For the reasons noted in the Langer Declaration and in view of the amendments above, Applicants respectfully submit that the rejections under 35 U.S.C. § 112, first paragraph, have been overcome, and respectfully request reconsideration and withdrawal of such rejections as they are applied to this application.

In section 10 at page 5 of the Detailed Action, claims 10-14 were rejected under 35 U.S.C. § 112, second paragraph, as being indefinite, with respect to the use of the terms "a cell of the specific immune system" in claims 10 and 11, a "cell of the unspecific immune system" as used in

claim 13, and a "degenerate cell of the immune system" as used in claim 14. Applicants respectfully disagree with and traverse these rejections.

With respect to these rejections under 35 U.S.C. § 112, second paragraph, the Examiner is directed to paragraphs 22-29 of the Langer Declaration. Dr. Langer explained that in view of his background, experience in the art to which this invention relates and knowledge of both spoken and written English and German (see Langer Declaration paragraphs 2-8), the terms to which the Examiner objected in claims 10-14 would be readily understood by those skilled in this art, particularly in view of the references he has cited in paragraphs 24-27 (including the priority parent application PCT/EP/00009, published as Publication No. WO 98/29540, the cover page and pages 21 and 73 being identified as Reference 19), with respect to "specific immunity" and "unspecific immunity," as well as a "degenerate cell of the immune system," which he specifically addressed in paragraph 29 and the references cited therein. Accordingly, Dr. Langer's conclusions in paragraphs 28 and 29 that these terms are well understood by those skilled in this art.

Moreover, with respect to claims 10-13, Applicants have amended claims 10, 11 and 13 to refer to the cells in a manner more typical of the way in which they are referred in the United States. Based on the same information in the Langer Declaration, the written description at page 25, lines 11-12, also has been amended to refer to the more common American terminology. No new matter has been added.

In view of the amendments and the evidence provided by the Langer Declaration and its cited references, Applicants respectfully request entry of all amendments and reconsideration and withdrawal of the rejections under 35 U.S.C. § 112, second paragraph.

Having addressed all aspects of the Office Action, Applicants respectfully request the reconsideration and withdrawal of all rejections and the issuance of a prompt Notice of Allowance.

Please note that Kristyne Bullock, the prior attorney of record, is no longer with the undersigned attorney's law firm. Accordingly, all further communications should be addressed to the undersigned attorney of record. The Examiner is encouraged to contact the undersigned attorney by telephone or e-mail if it is believed that doing so will help advance the prosecution of this application.

Respectfully submitted,

**JÜRGEN ECK, *ET AL.***

February 11, 2005 By: _____
(Date)

**ALAN S. NADEL**
Registration No. 27,363
**AKIN GUMP STRAUSS HAUER & FELD LLP**
One Commerce Square
2005 Market Street, Suite 2200
Philadelphia, PA 19103-7013
Telephone: 215-965-1200
**Direct Dial: 215-965-1280**
Facsimile: 215-965-1210
E-Mail: anadel@akingump.com

ASN/hg
Attachments:

Exhibit 1: Sambrook, *et al.*, title page and pages 2.114-2.117, 9.47-9.51 and 11.44-11.49.

Declaration of Martin Langer Under 37 C.F.R. § 1.132 (and curriculum vitae as Appendix A and references cited therein).

Enclosures:

Petition for Four-Month Extension of Time
Request for Continued Examination
Check in the amount of $1,565

1

# Molecular Cloning

## A LABORATORY MANUAL

## SECOND EDITION

J. Sambrook
UNIVERSITY OF TEXAS SOUTHWESTERN MEDICAL CENTER

E.F. Fritsch
GENETICS INSTITUTE

T. Maniatis
HARVARD UNIVERSITY

1890–1990 CSH CENTENNIAL
*Cold Spring Harbor Laboratory Press*
*1989*

### *Hybridization to Nitrocellulose Filters Containing Replicas of Bacteriophage λ Plaques*

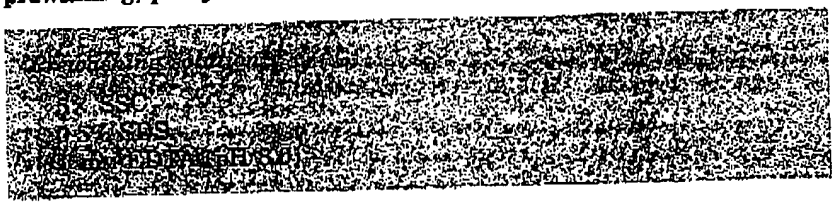
For a general discussion of the factors that affect the rate and specificity of hybridization of radioactive probes to nucleic acids immobilized on solid supports, see Chapter 9, pages 9.47–9.51.

The following protocol is designed for 30 circular filters, 82 mm in diameter. Appropriate adjustments should be made to the volumes when carrying out hybridization reactions with different numbers or sizes of filters.

1. Float the baked filters on the surface of a tray of 2 × SSC until they have become thoroughly wetted from beneath. Submerge the filters for 5 minutes.

2. *Optional:* Transfer the filters to a circular glass crystallizing dish containing 100 ml of prewashing solution. Stack the filters on top of one another in the solution, and cover the dish with Saran Wrap. In this and all subsequent steps, the filters should be slowly agitated on a rotating platform in a water bath or incubator at the appropriate temperature to prevent them from sticking to one another.

   **Important:** Do not allow the filters to dry at any stage during the prewashing, prehybridization, or hybridization steps.



   Incubate the filters for 1–2 hours at 42°C. Then, gently scrape the bacterial debris from the surfaces of the filters using Kimwipes soaked in the prewashing solution. This reduces background hybridization without affecting the intensity or sharpness of positive signals.

3. Transfer the filters to a glass crystallizing dish containing 60 ml of prehybridization solution. Incubate for 1–2 hours at the appropriate temperature (68°C when hybridization is to be carried out in aqueous solution; 42°C when hybridization is to be carried out in 50% formamide).

   Some workers prefer to incubate the filters in sealed plastic bags (Sears Seal-A-Meal or equivalent) (see, e.g., Chapter 9, page 9.53). This method avoids problems of evaporation and, because the sealed bags can be submerged in a water bath, ensures that the temperatures during hybridization and washing are correct. The bags must be opened and resealed when changing buffers. To avoid radioactive contamination of the water bath, the resealed bag containing radioactivity should be sealed inside a second, noncontaminated bag.

   Often, small bubbles of air form on the surface of the filter as the temperature of the prehybridization solution increases. It is important that these bubbles be removed by occasionally agitating the fluid in the bag; otherwise, the components of the prehybridization solution will not be able to coat the filter evenly. This problem can be minimized by heating the prehybridization solution to the appropriate temperature before adding it to the bag.

   The filters should be completely covered by the prehybridization solution. During

prehybridization, sites on the nitrocellulose filter that nonspecifically bind single- or double-stranded DNA become blocked by proteins in the BLOTTO.

When $^{32}$P-labeled cDNA or RNA is used as a probe, poly(A) at a concentration of 1 μg/ml should be included in the prehybridization and hybridization solutions to prevent the probe from binding to T-rich sequences that are found fairly commonly in eukaryotic DNA.

Whether or not to use a prehybridization solution containing formamide is largely a matter of personal preference. Both versions of these solutions give excellent results and neither has clear-cut advantages over the other. However, hybridization in 50% formamide at 42°C is less harsh on nitrocellulose filters than is hybridization at 68°C in aqueous solution. Offsetting this advantage is the two- to threefold slower rate of hybridization in solutions containing formamide.

To maximize the rate of annealing of the probe with its target, hybridizations are usually carried out in solutions of high ionic strength (6× SSC or 6× SSPE) at a temperature that is 20–25°C below $T_m$ (see Chapter 9, pages 9.50–9.51). Both solutions work equally well when hybridization is carried out in aqueous solvents. However, when formamide is included in the hybridization buffer, 6× SSPE is preferred because of its greater buffering power.

*Prehybridization solution*

*Either*
50% formamide
6× SSC (or 6× SSPE)
0.05× BLOTTO
*or*
6× SSC (or 6× SSPE)
0.05× BLOTTO

*Formamide:* Many batches of reagent-grade formamide are sufficiently pure to be used without further treatment. However, if any yellow color is present, the formamide should be deionized by adding Dowex XG8 mixed-bed resin and stirring on a magnetic stirrer for 1 hour and filtering twice through Whatman No. 1 paper. Deionized formamide should be stored in small aliquots under nitrogen at −70°C.

*1× BLOTTO:* Boving Lacto-Transfer Technique Optimizer (Johnson et al. 1984) is 5% nonfat dried milk dissolved in water containing 0.02% sodium azide. It should be stored at 4°C. 1× BLOTTO is as effective a blocking agent as Denhardt's reagent, but much less expensive. It should not be used in combination with high concentrations of SDS, which will cause milk proteins to precipitate. If background hybridization is a problem, NP-40 may be added to the hybridization solution to a final concentration of 1%. BLOTTO should not be used as a blocking agent when radiolabeled RNA is used as a hybridization probe, because of the possibility that the dried milk may contain significant amounts of RNAase activity.

**Caution:** Sodium azide is poisonous. It should be handled with great care wearing gloves, and solutions containing it should be clearly marked.

4. Denature $^{32}$P-labeled double-stranded DNA probe by heating for 5 minutes to 100°C. Chill the probe rapidly in ice water. Single-stranded probe need not be denatured. Add the probe to the prehybridization solution covering the filters. Incubate at the appropriate temperature until $1-3 \times C_o t_{1/2}$ is achieved (see Chapter 9, page 9.48). During the hybridization, the containers holding the filters should be tightly closed to prevent loss of fluid by evaporation.

   Alternatively, the probe may be denatured by adding 0.1 volume of 3 N NaOH. After 5 minutes at room temperature, transfer the probe to ice water and add 0.05 volume of 1 M Tris · Cl (pH 7.2) and 0.1 volume of 3 N HCl. Store the probe in ice water until it is needed.

   Between $2 \times 10^5$ and $1 \times 10^6$ cpm of $^{32}$P-labeled probe (sp. act. $\geq 5 \times 10^7$ cpm/$\mu$g) should be used per milliliter of prehybridization solution. Using more probe will cause the background of nonspecific hybridization to increase; using less will reduce the rate of hybridization.

5. When the hybridization is completed, remove the hybridization solution and immediately immerse the filters in a large volume (300–500 ml) of a solution of 2× SSC and 0.1% SDS at room temperature. Agitate the filters gently, and turn them over at least once during washing. After 5 minutes, transfer the filters to a fresh batch of wash solution and continue to agitate them gently. Repeat the washing procedure twice more. At no stage during the washing procedure should the filters be allowed to dry.

   Hybridization mixtures containing radiolabeled single-stranded probes may be stored at 4°C for several days and reused without further treatment. However, hybridization mixtures containing complementary strands of DNA should be discarded, since there is no satisfactory way to denature the double-stranded DNA that forms during the first round of hybridization.

6. Wash the filters twice for 1–1.5 hours in 300–500 ml of a solution of 1 × SSC and 0.1% SDS at 68°C. At this point, the background is usually low enough to put the filters on film. If the background is still high or if the experiment demands washing at high stringencies, immerse the filters for 60 minutes in 300–500 ml of a solution of 0.2 × SSC and 0.1% SDS at 68°C.

7. Dry the filters in the air on paper towels at room temperature. Arrange the filters (numbered side up) on a sheet of Saran Wrap. Apply adhesive dot labels marked with radioactive ink to several asymmetric locations on the Saran Wrap. These markers serve to align the autoradiograph with the filters. Cover the labels with Scotch Tape. This prevents contamination of the film holder or intensifying screen with radioactive ink.

   Radioactive ink is made by mixing a small amount of $^{32}$P with waterproof black drawing ink. We find it convenient to make the ink in three grades: very hot (>2000 cps on a hand-held minimonitor), hot (>500 cps on a hand-held minimonitor), and cool (>50 cps on a hand-held minimonitor). Use a fiber-tip pen to apply ink of the desired hotness to the adhesive labels. Attach radioactive-warning tape to the pen, and store it in an appropriate place.

8. Cover the filters with a second sheet of Saran Wrap. Expose the filters to X-ray film (Kodak XAR or equivalent) for 12–16 hours at −70°C with an intensifying screen (see Appendix E).

9. Develop the film and align it with the filters using the marks left by the radioactive ink. Use a nonradioactive fiber-tip pen to mark the film with the positions of the asymmetrically located dots on the numbered filters. Tape a piece of clear Mylar or other firm transparent sheet to the film. Mark on the clear sheet the positions of positive hybridization signals. Also mark (in a different color) the positions of the asymmetrically located dots. Remove the clear sheet from the film. Identify the positive plaques by aligning the dots on the clear sheet with those on the agar plate.

   Some batches of nitrocellulose filters swell and distort during hybridization and subsequent drying, so that it becomes difficult to align the two sets of dots. This problem can be alleviated to some extent by autoclaving the dry filters between pieces of damp Whatman 3MM paper before use (10 lb/sq. in. for 10 minutes on liquid cycle). Nylon membranes do not suffer from this problem.

10. Each positive plaque should be picked as described on page 2.63 and placed in 1 ml of SM containing a drop of chloroform. Often, the alignment of the filters with the plate does not permit identification of an individual hybridizing plaque. In this case, an agar plug containing several plaques should be picked. An aliquot (usually 50 $\mu$l of a $10^{-2}$ dilution) of the bacteriophages that elute from the agar plug is replated so as to obtain approximately 500 plaques on a 90-mm plate. These plaques are then screened a second time by hybridization. A single, well-isolated positive plaque should be picked from the secondary screen and used to make a plate stock (see page 2.65 or 2.66).

## HYBRIDIZATION OF RADIOLABELED PROBES TO IMMOBILIZED NUCLEIC ACIDS

There are many methods available to hybridize radioactive probes in solution to nucleic acids immobilized on solid supports such as nitrocellulose filters or nylon membranes. These methods differ in the following respects:

- Solvent and temperature used (e.g., 68°C in aqueous solution or 42°C in 50% formamide)
- Volume of solvent and length of hybridization (large volumes for periods as long as 3 days or minimal volumes for periods as short as 4 hours)
- Degree and method of agitation (continuous shaking or stationary)
- Use of agents such as Denhardt's reagent or BLOTTO to block the nonspecific attachment of the probe to the surface of the solid matrix
- Concentration of the labeled probe and its specific activity
- Use of compounds, such as dextran sulfate (Wahl et al. 1979) or polyethylene glycol (Renz and Kurz 1984; Amasino 1986), that increase the rate of reassociation of nucleic acids
- Stringency of washing following the hybridization

Although the choice depends to a large extent on personal preference, we offer the following guidelines for choosing among the various methods available.

1. Hybridization reactions in 50% formamide at 42°C are less harsh on nitrocellulose filters than is hybridization at 68°C in aqueous solution. However, it has been found that the kinetics of hybridization in 80% formamide are approximately four times slower than in aqueous solution (Casey and Davidson 1977). Assuming a linear relationship between the rate of hybridization and the formamide concentration, the rate in 50% formamide should be two to three times slower than the rate in aqueous solution. Both types of solvents give excellent results and neither has a clear-cut advantage over the other.
2. The smaller the volume of hybridization solution, the better. In small volumes of solution, the kinetics of nucleic acid reassociation are faster and the amount of probe needed can be reduced so that the DNA on the filter acts as the driver for the reaction. However, it is essential that sufficient liquid be present for the filters to remain covered at all times by a film of the hybridization solution.
3. Continual movement of the probe solution across the filter is unnecessary, even for a reaction driven by the DNA immobilized on the filter. However, if a large number of filters are hybridized simultaneously, agitation is advisable to prevent the filters from adhering to one another.
4. The kinetics of the hybridization reaction are difficult to predict from theoretical considerations, partly because the exact concentration of the

immobilized nucleic acid and its availability for hybridization are unknown. When using probes that have the capacity to self-anneal (e.g., nick-translated double-stranded DNA), the following rule of thumb is useful: Allow the hybridization to proceed for a time sufficient to enable the probe in solution to achieve $1–3 \times C_o t_{1/2}$. In 10 ml of hybridization solution, 1 $\mu$g of a probe of 5-kb complexity will reach $C_o t_{1/2}$ in 2 hours. To determine the time of half-renaturation for any other probe, simply enter the appropriate values into the following equation:

$$1/x \times y/5 \times z/10 \times 2 = \text{number of hours to achieve } C_o t_{1/2}$$

where $x$ = the weight of the probe added (in micrograms), $y$ = its complexity (for most probes, complexity is proportional to the length of the probe in kilobases), and $z$ = the volume of the reaction (in milliliters).

After hybridization to $3 \times C_o t_{1/2}$ has been reached, the amount of probe available for additional hybridization to the filter is negligible. For probes that do not have the capacity to self-anneal (e.g., RNA probes synthesized in vitro by bacteriophage-encoded DNA-dependent RNA polymerases; see Chapter 10), the hybridization time may be shortened, since the lack of a competing reaction in the solution favors hybridization of the probe to the DNA immobilized on the filter.

5. Several different types of agents can be used to block the nonspecific attachment of the probe to the surface of the filter. These include Denhardt's reagent (Denhardt 1966), heparin (Singh and Jones 1984), and nonfat dried milk (Johnson et al. 1984). Frequently, these agents are used in combination with denatured, fragmented salmon sperm or yeast DNA and detergents such as SDS. In our experience, virtually complete suppression of background hybridization is obtained by prehybridizing filters with a blocking agent consisting of 5× Denhardt's reagent, 0.5% SDS, and 100 $\mu$g/ml denatured, fragmented DNA. We recommend this mixture whenever the signal-to-noise ratio is expected to be low, for example, when carrying out northern analysis of low-abundance mRNAs or Southern hybridizations with single-copy sequences of mammalian DNA. However, in most other circumstances (Grunstein/Hogness hybridization [1975], Benton/Davis hybridization [1977], Southern hybridization [1975] of abundant DNA sequences, etc.), we recommend using 0.25% nonfat dried milk (0.05× BLOTTO; Johnson et al. 1984). This is much less expensive, easier to use than Denhardt's reagent, and, for these purposes, gives results that are equally satisfactory. In general, Denhardt's reagent is more effective for nylon membranes. The signal-to-noise ratio obtained with most brands of nylon membranes is higher with Denhardt's reagent than with BLOTTO. Nonfat dried milk is not recommended when using RNA probes or when carrying out northern hybridizations because of the possibility that it might contain unacceptably high levels of RNAase activity. For more information about blocking agents, see Table 9.1.

6. Blocking agents are usually included in both the prehybridization and hybridization solutions when nitrocellulose filters are used. However, when the nucleic acid is immobilized on nylon membranes, the blocking agents are often omitted from the hybridization solution, since high

concentrations of protein are believed to interfere with the annealing of the probe to its target. This quenching of the hybridization signal is particularly noticeable when oligonucleotides or probes less than 100 nucleotides in length are used.

7. In the presence of 10% dextran sulfate or 10% polyethylene glycol, the rate of hybridization is accelerated approximately tenfold (Wahl et al. 1979; Renz and Kurz 1984; Amasino 1986) because nucleic acids are excluded from the volume of the solution occupied by the polymer and their effective concentration is therefore increased. Although dextran sulfate and polyethylene glycol are useful in circumstances where the rate of hybridization is the limiting factor in detecting rare sequences (e.g., northern or genomic Southern blots), they are of no benefit when screening bacterial colonies or bacterial plaques. In addition, they can sometimes lead to high backgrounds, and hybridization solutions containing them are always difficult to handle because of their viscosity. We therefore recommend that dextran sulfate and polyethylene glycol not be used unless the rate of hybridization is very slow, the filter contains very small amounts of DNA, or the amount of radiolabeled probe is limiting.

8. To maximize the rate of annealing of the probe with its target, hybridizations are usually carried out in solutions of high ionic strength (6 × SSC or 6 × SSPE) at a temperature that is 20–25°C below the melting temperature ($T_m$). Both solutions work equally well when hybridization is carried out in aqueous solvents. However, when formamide is included in the hybridization buffer, 6 × SSPE is preferred because of its greater buffering power.

9. In general, the washing conditions should be as stringent as possible (i.e., a combination of temperature and salt concentration should be chosen that is approximately 12–20°C below the calculated $T_m$ of the hybrid under study). The temperature and salt conditions can often be determined empirically in preliminary experiments in which samples of genomic DNA immobilized on filters are hybridized to the probe of interest and then washed under conditions of different stringencies.

10. To minimize background problems, it is best to hybridize for the shortest possible time using the minimum amount of probe. For Southern hybridization of mammalian genomic DNA where each lane of the gel contains 10 $\mu$g of DNA, 10–20 ng/ml radiolabeled probe (sp. act. = $10^9$ cpm/$\mu$g or greater) should be used and hybridization should be carried out for 12–16 hours at 68°C in aqueous solution or for 24 hours at 42°C in 50% formamide. For Southern hybridization of fragments of cloned DNA where each band of the restriction digest contains 10 ng of DNA or more, much less probe is required. Typically, hybridization is carried out for 6–8 hours using 1–2 ng/ml radiolabeled probe (sp. act. = $10^9$ cpm/$\mu$g or greater).

11. *Useful facts*:

    a. The $T_m$ of the hybrid formed between the probe and its target may be estimated from the following equation (Bolton and McCarthy 1962):

$$T_m = 81.5°C + 16.6(\log_{10}[Na^+]) + 0.41(\text{fraction G + C}) - 0.63(\%\ \text{formamide}) - (600/l)$$

where $l$ = the length of the hybrid in base pairs.

This equation is valid for:

- Concentrations of $Na^+$ in the range of 0.01 M to 0.4 M. It predicts $T_m$ less accurately in solutions of higher $[Na^+]$.
- DNAs whose G + C content is in the range of 30% to 75%. Note that the depression of $T_m$ in solutions containing formamide is greater for poly(dA:dT) (0.75°C/1% formamide) and less for DNAs rich in poly(dG:dC) (0.50°C/1% formamide) (Casey and Davidson 1977).

The equation applies to the "reversible" $T_m$ that is defined by optical measurement of hyperchromicity at $OD_{267}$. The "irreversible" $T_m$, which is more important for autoradiographic detection of DNA hybrids, is usually 7–10°C higher than that predicted by the equation.

Similar equations have been derived for:

i. RNA probes hybridizing to immobilized RNA (Bodkin and Knudson 1985)

$$T_m = 79.8°C + 18.5(\log_{10}[Na^+]) + 0.58(\text{fraction G + C}) + 11.8(\text{fraction G + C})^2 - 0.35(\%\ \text{formamide}) - (820/l)$$

ii. DNA:RNA hybrids (Casey and Davidson 1977)

$$T_m = 79.8°C + 18.5(\log_{10}[Na^+]) + 0.58(\text{fraction G + C}) + 11.8(\text{fraction G + C})^2 - 0.50(\%\ \text{formamide}) - (820/l)$$

Comparison of these equations shows that the relative stability of nucleic acid hybrids decreases in the following order: RNA:RNA (most stable), RNA:DNA (less stable), and DNA:DNA (least stable). In aqueous solutions, the $T_m$ of a DNA:DNA hybrid is approximately 10°C lower than that of the equivalent RNA:RNA hybrid. In 80% formamide, the $T_m$ of an RNA:DNA hybrid is approximately 10°C higher than that of the equivalent DNA:DNA hybrid.

b. The $T_m$ of a double-stranded DNA decreases by 1–1.5°C with every 1% decrease in homology (Bonner et al. 1973).

The above equations apply only to hybrids greater than 100 nucleotides in length. The behavior of oligonucleotide probes is described in detail in Chapter 11.

For a general discussion of hybridization of nucleic acids bound to solid supports, see Meinkoth and Wahl (1984).

## Conditions for Hybridization of Oligonucleotide Probes

When using oligonucleotides as probes, the aim is to find conditions that are stringent enough to guarantee specificity and sufficiently flexible to allow formation of stable hybrids at an acceptable rate. For DNA molecules more than 200 nucleotides in length, hybridization is usually carried out at 15–25°C below the calculated melting temperature ($T_m$) of a perfect hybrid. However, as the length of the probe is decreased, the $T_m$ is lowered to the point where it is often impractical to carry out hybridization at $T_m - 25$°C. Typically, therefore, hybridization with synthetic oligonucleotides is carried out under conditions that are only 5–10°C below the $T_m$. Although such stringent conditions reduce the number of mismatched clones that are detected with short oligonucleotide probes, they have the less desirable consequence of reducing the rate at which perfect hybrids form.

Hybrids formed between DNA molecules more than 200 nucleotides in length are completely stable for all practical purposes. The chances that such a long stretch of double helix will unwind at temperatures 15–25°C below the $T_m$ are extremely small. However, hybrids (even perfect hybrids) formed between short oligonucleotides and their target sequences at 5–10°C below the $T_m$ are far easier to unwind, and hybridization reactions of this type can be regarded as reversible. This has important practical consequences. Whereas hybrids formed between longer DNA molecules are essentially stable under the conditions used for posthybridization washing, hybrids (even perfect hybrids) involving short oligonucleotides are not. Posthybridization washing of such hybrids must therefore be carried out rapidly so that the probe does not dissociate from its target sequence. For this reason, hybridizations with short oligonucleotides should be carried out under stringent conditions (5–10°C below the $T_m$) using high concentrations (0.1–1.0 pmole/ml) of probe. When only one or a small number of oligonucleotides (<8) are used as probes, the annealing reaction rapidly reaches equilibrium, and hybridization should therefore be terminated after 3 or 4 hours. More complex mixtures, in which the concentration of each oligonucleotide is comparatively low, require hybridization to be carried out for proportionately longer periods. For example, mixtures of 32 or more oligonucleotides should be hybridized for 1–2 days. Posthybridization washing should be carried out for brief periods of time, initially under conditions of low stringency and then under conditions of stringency equal to those used for hybridization.

## CALCULATING MELTING TEMPERATURES FOR PERFECTLY MATCHED HYBRIDS BETWEEN OLIGONUCLEOTIDES AND THEIR TARGET SEQUENCES

When using single oligonucleotides that match the target sequence perfectly, hybridization conditions can easily be derived from the calculated $T_m$ of the hybrid. For oligonucleotides shorter than 18 nucleotides, the $T_m$ of the hybrid can be estimated by multiplying the number of A + T residues in the hybrid by 2°C and the number of G + C residues by 4°C and adding the two numbers (Itakura et al. 1984). However, this method overestimates the $T_m$ of hybrids involving longer oligonucleotides.

A different approach has been taken by E. Fritsch (unpubl.), who found that the equation originally used to calculate the relationship between G + C content, ionic strength of the hybridization solution, and the $T_m$ of long DNA molecules (Bolton and McCarthy 1962):

$$T_m = 81.5 + 16.6(\log_{10}[Na^+]) + 0.41(\text{fraction G} + \text{C}) - (600/N),$$

where $N$ = chain length, predicts reasonably well the $T_m$ for oligonucleotides as long as 60–70 nucleotides and as short as 14 nucleotides.

This formula only works for $Na^+$ concentrations of 1 M or less.

## HYBRIDIZATION OF POOLS OF OLIGONUCLEOTIDES

It is easy to calculate accurately the $T_m$ of a perfectly matched hybrid formed between a single oligonucleotide and its target sequence. However, when using pools of oligonucleotides whose members have greatly different contents of G + C, it is impossible to estimate a consensus $T_m$. Because it is not possible to know which member of the pool will match the target sequence perfectly, conditions must be used that allow the oligonucleotide with the lowest content of G + C to hybridize efficiently. Usually, conditions are chosen to be 2°C below the calculated $T_m$ of the most A/T-rich member of the pool (Suggs et al. 1981b). However, the use of such "lowest common denominator" conditions can lead to a number of false positives, because mismatched hybrids formed by oligonucleotides of higher G + C content may be more stable than a perfectly matched hybrid formed by the correct oligonucleotide. In most cases, this problem is not serious, since the number of positive clones obtained by screening cDNA libraries with pools of oligonucleotides is usually quite manageable. It is therefore possible to easily distinguish false positives from true positives by another test (e.g., DNA sequencing or hybridization with a second pool of oligonucleotides corresponding to another segment of amino acid sequence).

In those cases when the number of positives is unacceptably high, it may be worthwhile to consider using hybridization solvents that contain the quaternary alkylammonium salts tetraethylammonium chloride (TEACl) or tetramethylammonium chloride (TMACl) instead of sodium chloride (Melchior and von Hippel 1973; Jacobs et al. 1985, 1988; Wood et al. 1985; Gitschier et al. 1986; Wozney 1989). In these solvents, the $T_m$ of a hybrid is independent of its base composition and dependent primarily on its length. Thus, by choosing a temperature for hybridization appropriate for the lengths of the oligonucleotides in a pool, the effects of potential mismatches can be minimized.

It is important to obtain an accurate estimate of the $T_m$ in TMACl or TEACl before using pools of oligonucleotides to screen cDNA or genomic DNA libraries. Jacobs et al. (1988) measured the $T_i$ (the irreversible melting temperature of the hybrid formed between the probe and its target sequence) as a function of chain length for a number of oligonucleotides of differing G + C content in solvents containing either sodium or tetramethylammonium ions. Hybrids involving oligonucleotides 16 and 19 nucleotides in length melt over a smaller range of temperature in solvents containing TMACl than in solvents containing sodium salts (3°C for TMACl vs. 17°C for SSC when hybridizing 16-mers; 5°C for TMACl vs. 20°C for SSC when hybridizing 19-mers). For 14-mers, the effect is much less dramatic (7°C for TMACl vs. 9°C for SSC). Similar, but less extensive, data are available for solvents containing TEACl (Jacobs et al. 1988).

The optimal temperature for hybridization is usually chosen to be 5°C below the $T_i$ for the given chain length. The recommended hybridization temperature for 17-mers in 3 M TMACl is 48–50°C; for 19-mers, it is 55–57°C; and for 20-mers, it is 58–66°C. Three points are worth emphasizing. First, the $T_i$s of hybrids are uniformly 15–20°C higher in solvents containing TMACl than in solvents containing TEACl. The higher $T_i$ in solvents containing TMACl allows hybridization to be performed at temperatures that

suppress nonspecific adsorption of the probe to solid supports (such as nylon membranes), resulting in lower nonspecific backgrounds. Second, hybridization solvents containing TMACl do not have significant advantages over those containing sodium ions until the length of the oligonucleotide exceeds 16 nucleotides. Finally, the data have been extensively examined for 16-mers, 19-mers, and, in previous studies, for much longer DNA molecules (Melchior and von Hippel 1973). It is currently an untested assumption that the same beneficial effect will be seen for DNA molecules of all intermediate lengths.